Basolateral to Apical Transcytosis in Polarized Cells Is Indirect and Involves BFA and Trimeric G Protein Sensitive Passage through the Apical Endosome

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Abstract. We have used temperature and nocodazole blocks in an in vivo basolateral to apical transcytosis assay to dissociate the early transcytotic steps occurring during the formation of transcytotic vesicles and their microtubule-dependent translocation into the apical region, from the late steps when transcytotic cargo is delivered into the apical media. We found that polarized MDCK cells transfected with rabbit polymeric IgA receptor (pIgA-R) internalize basolaterally added pIgA-R ligand ($[Fab]_2$ fragment of IgG against the receptor's ectodomain) at 17°C but do not deliver it to the apical PM. Instead, the ligand accumulates in an apically localized transcytotic compartment, distal to the basolateral endosome and the microtubule-requiring translocation step. We have characterized this compartment and show that it is distinct from basolateral transferrin recycling endosomes, basolateral early endosomes or late endosomes or lysosomes. The apical transcytotic compartment colocalizes with the compartment containing apically recycling membrane markers (ricin and apically internalized pIgA-R ligand) but is distinct from the compartment receiving apically internalized fluid phase marker (BSA). This compartment is an intermediate station of the overall pathway since transcytotic ligand can exit the compartment and be released into the apical medium when cells preloaded at 17°C are subsequently incubated at 37°C.

We have used this system to examine the effect of Brefeldin A (BFA) and the involvement of trimeric GTPases in the late (post apical transcytotic compartment) steps of the transcytotic pathway. We found that addition of BFA or cholera toxin, a known activator of $Gs\alpha$, to cells preloaded with transcytotic ligand at 17°C significantly inhibits the exit of ligand from the apical transcytotic compartment. General structure and function of the apical endosome are not affected since neither BFA nor cholera toxin inhibit the recycling of apically internalized membrane markers (ricin and pIgA-R ligand) from the same compartment.

The data suggest that transcytosis connects the "membrane-sorting" sub-domain of the basolateral endosome with a homologous sub-domain of the apical endosome and that exit of transcytosing cargo from the apical endosome is controlled by a BFA and trimeric G protein sensitive mechanism, distinct from that used for recycling of apically internalized proteins (ricin or pIgA-R).

POLARIZED epithelial cells possess two functionally, morphologically, and biochemically distinct plasma membrane (PM)[1] domains (for review see Simons and Fuller, 1985). Connection between the basolateral and apical domain is mediated by *transcytosis*, a multi-step vesicular transport pathway. Depending on the cell type and the marker protein followed, transcytosis can occur predominantly from basolateral to apical PM (e.g., pIgA and pIgA-R in liver; Sztul et al., 1983; Hoppe et al., 1985), from apical to basolateral PM (e.g., IgG in enterocytes of neonatal rats; Rodewald and Abrahamson, 1980; Rodewald and Kraenbuhl, 1984), or equally from both surfaces (e.g., fluid-phase marker in MDCK cells; Bomsel et al., 1989).

Basolateral to apical transcytosis has been extensively studied in rat liver and in polarized MDCK cells by examining the transcytotic traffic of a transmembrane protein, the pIgA-R (Mullock et al., 1979; Renston et al., 1980; Geuze et al., 1984; Hoppe et al., 1985; Limet et al., 1985; Mostov and Deitcher, 1986; Breitfeld et al., 1989). Transcytotic pathway can be subdivided into several spatially and/or temporally distinct steps: (*a*) internalization of transcytotic cargo from the basolateral surface via clathrin-coated pits and vesicles; (*b*) delivery to the basolateral early/sorting en-

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1. *Abbreviations used in this paper*: ARF, ADP-ribosylation factor; BFA, Brefeldin A; BSA-FITC, FITC-labeled BSA; pIgA-R, polymeric IgA receptor; PM, plasma membrane; TCVs, transcytotic vesicles.

dosome; (*c*) active sorting from other transmembrane and content proteins and segregation into a distinct endosomal sub-domain; (*d*) budding of transcytotic vesicles (TCVs), from that sub-domain; (*e*) translocation of TCVs into the subapical region of cells via a microtubule-dependent mechanism; and (*f*) subsequent delivery of transcytotic cargo to the apical PM (Geuze et al., 1984; Hoppe et al., 1985; Breitfeld et al., 1989, 1990; Casanova et al., 1990; Hunziker et al., 1990). Although the initial stages of the transcytotic pathway, namely those before the formation of TCVs, are well documented, the later steps, i.e., those occurring within the apical region of cells, are poorly defined. One of the major questions is whether TCVs deliver their cargo *directly* by fusing with the apical PM or whether the delivery is *indirect* and involves fusion of TCVs with other compartments, from which transcytotic cargo is subsequently delivered to the apical PM.

Relevant to such considerations is the finding that polarized MDCK cells contain distinct endosomal systems: one set of early/sorting endosomes is localized close to the basolateral PM and receives cargo endocytosed only from that PM domain and another, close to the apical PM which receives cargo only from the apical domain. Cargo internalized into these distinct sorting endosomes does not mix, suggesting that fusion between the two sets of endosomal compartments is inhibited (Bomsel et al., 1989; Parton et al., 1989). Both of the endosomal systems "empty" into a common late endosomal system in which cargo internalized from basolateral and apical surface mixes before delivery to lysosomes (Bomsel et al., 1989; Hughson and Hopkins, 1990). The presence of distinct early endosomal populations in polarized cells raises an interesting question: if transcytosis occurs *indirectly*, is it possible that TCVs act as the vesicular link connecting the two endocytic compartments?

To address this question we examined the compartments involved in the late steps of the transcytotic pathway by analyzing the basolateral-to-apical transcytosis of pIgA-R pseudo-ligand ($[Fab]_2$ fragments of IgG directed against the pIgA-R ectodomain) in polarized MDCK cells transfected with rabbit pIgA-R (Mostov and Deitcher, 1986). To focus exclusively on late stages, we used an in vivo treatment which dissociates events occurring before the delivery of cargo into the apical region from the later steps of the pathway. Specifically, we took advantage of the observation by Hunziker et al. (1990) that incubation of MDCK cells at 17°C results in a partial block of the transcytotic pathway. At this temperature, pIgA-R ligand is endocytosed, sorted from non-transcytotic ligands into TCVs which are then translocated on microtubules into the apical region of cells (Hunziker et al., 1990; Breitfeld et al., 1990). However, during 17°C incubations, delivery of the pIgA-R ligand into the apical media is inhibited (Hunziker et al., 1990) and the ligand accumulates in an undefined apical compartment. The release of apically accumulated pIgA-R ligand into the apical medium can be induced by shifting the cells to 37°C and is not microtubule-dependent.

We used this temperature block to functionally characterize the 17°C apical transcytotic compartment and to study the mechanism controlling the movement of transcytotic cargo from that compartment to the cell surface. Specifically, we tested whether addition of BFA or activation of trimeric G proteins influences the traffic of pIgA-R ligand from the apical transcytotic compartment to the apical PM.

BFA has been shown to dramatically modulate membrane traffic within the secretory and endocytic pathways (for review see Klausner et al., 1992). It inhibits the guanine nucleotide exchange on ADP-ribosylation factor (ARF) and thus prevents its binding to the membrane where ARF is required to promote the association of cytosolic coatomer and other proteins (Waters et al., 1991; Narula et al., 1992; Klausner et al., 1992; Donaldson et al., 1992; Randazzo et al., 1993; Palmer et al., 1993). Membrane "coats" might be involved in protein sorting or stabilizing proteins within a specific membrane sub compartment in a manner analogous to the action of adaptins during clathrin-mediated membrane traffic or in preventing incorrect membrane fusion events (Pearse and Robinson, 1990; Rothman and Orci, 1992). The effect of BFA on the transcytotic pathway in MDCK cells has been examined previously and, as shown by Hunziker et al. (1991), the drug inhibits the transcytotic delivery of pIgA-R physiological ligand, dimeric IgA (dIgA), into the apical media. The BFA induced block is early (ligand is not translocated into the apical region of cells) and is presumably due to the inability of pIgA-R to be correctly sorted from the basolateral endosome into the transcytotic pathway (Hunziker et al., 1993). Effects of BFA on later steps of transcytosis have not been examined.

Trimeric GTPases were originally identified as members of the signal transduction cascade, functionally coupling PM receptors (e.g., rhodopsin and β-adrenergic receptor) to effectors (cGMP; phosphodiesterase and adenylyl cyclase, respectively) (Kaziro et al., 1991). However, it is now clear that they perform additional functions, including the regulation of the secretory and endocytic pathways (Barr et al., 1991; Stow et al., 1991; Colombo et al., 1992; Leyte et al., 1992; Carter et al., 1993). Trimeric GTPases appear to cross-talk with BFA-responsive elements since they also modulate the membrane association of specific coat proteins, for example, the coatomer (present in Golgi-derived non-clathrin-coated vesicles), ARF (a compartment of clathrin and non-clathrin-coated vesicles), and γ-adaptin (found in HA1/AP1 adaptor complex of the TGN-derived clathrin-coated vesicles) (Serafini et al., 1991; Donaldson et al., 1991; Ktistakis et al., 1992; Robinson and Kreis, 1992; Wong and Brodsky, 1992). It has been shown that activation of specific trimeric GTPases decreases ARF binding to membranes and thus can influence coat assembly and membrane traffic (for review see Bomsel and Mostov, 1992).

We have shown previously that rat liver TCVs contain a major 108-kD protein associated with their cytosolic surface and that this protein exists in cytosolic and TCV membrane associated pools as a hexameric complex of ~600 kD (Sztul et al., 1991; Gow et al., 1993). More recently, we have found that the association of this complex with the TCV membrane is regulated by a cholera toxin responsive Gs (Barroso, M., and E. S. Sztul, manuscript in preparation), suggesting that trimeric GTPases might control transcytotic traffic in a manner analogous to their role in other membrane traffic, i.e., by modulating the membrane association of specific coat components. Involvement of trimeric Gs in early stages of transcytosis has been recently documented (Bomsel and Mostov, 1993) but the role of trimeric GTPases during late stages of transcytosis has not been reported.

In this paper, we show that basolateral to apical transcytosis of pIgA-R ligand occurs *indirectly* by transport from the basolateral endosome to an apical endosomal compartment

and that the transcytotic compartment colocalizes with a specific sub-domain of the apical endosome, namely that involved in apical membrane recycling. We also show that addition of BFA and activation of trimeric G proteins modulate late steps of the transcytotic pathway, i.e., those occurring after transcytosed cargo is delivered to the apical endosome but before its exit from that compartment. Since neither BFA nor activation of trimeric G proteins inhibits apical membrane recycling through the same apical endosome, it appears that a sorting mechanism sensitive to BFA and involving G proteins controls the exit of transcytotic cargo from the apical transcytotic compartment into the apical recycling pathway.

Materials and Methods

Materials

Nocodazole, ricin-FITC, and ricin-HRP were purchased from Sigma Chem. Co. (St. Louis, MO). FITC was obtained from Molecular Probes, Inc. (Eugene, OR). [^{3}H]Sodium borohydride with a specific activity of 50.4 Ci/mmol was obtained from New England Nuclear (Boston, MA). Cy3 fluorochrome was purchased from Biological Detections Systems. Affinity-purified anti-goat IgG was from Cappel Labs. (Cochranville, PA). Anti-goat [Fab]$_2$ were purchased from Jackson ImmunoResearch Labs., Inc. (West Grove, PA).

Culture of MDCK Cells

MDCK cells expressing rabbit pIgA-R (kindly provided by Dr. K. Mostov, UCSF) were grown in polycarbonate filters (0.4 μm pore size; 24-mm diam) fitted within Transwell chambers (Costar Corp., Cambridge, MA) or in tissue culture inserts (Nunc, Inc., Naperville, IL) which use Anapore membranes as a cell substrate. These membranes have the advantage of being transparent when wet. No difference was observed in the results obtained from cells grown on either membranes. The cells were cultured at 37°C, in 5% CO_2 atmosphere and in DMEM containing 10% FBS, penicillin and streptomycin. One confluent 100-mm plate was used to plate a 6-well Transwell and cells were used for transcytosis experiments after 3 d of growth. Nunc tissue culture inserts were used in 24-well plates, under the same plating conditions. As shown by Podbilewicz and Mellman (1990), apical internalization of radiolabeled dog holo-transferrin was <5% of basolateral internalization, indicating that MDCK cells form polarized PM domains 3 d after plating.

In Vivo Transcytosis Assay

[Fab]$_2$ fragments of antibodies raised against the extracellular domain (secretory component) of the rabbit pIgA-R were used as a reporter molecule (Breitfeld et al., 1989). Affinity-purified immunoglobulins were subjected to pepsin digestion to generate [Fab]$_2$ fragments. The 110-kD fragments were then radiolabeled using tritiated sodium borohydride (Rice and Means, 1971). A typical preparation had a specific activity of 1-2 × 10^6 cpm/μg.

Radiolabeled [Fab]$_2$ fragments were added (5 μg/ml) to the basolateral media of the Transwell chambers in DMEM containing 20 mM Hepes, pH 7.3, and 0.5% BSA. After internalization at 17°C for 4 h, cells were washed extensively with PBS^{++} (containing Mg^{2+} and Ca^{2+}) and further incubated with nocodazole (33 μM) during 1 h on ice, to depolymerize the microtubular network (Hunziker et al., 1990). Cells were then incubated at 37°C for different periods of time in the presence or absence of nocodazole.

Immunoprecipitation with affinity-purified anti-goat IgG and protein A-Sepharose was used to recover the [Fab]$_2$ fragments released into the apical media. The immunoprecipitates and the remaining filters were counted, and the amount of label recovered represents the ligand internalized into the transcytotic pathway. To show that the transport of the [Fab]$_2$ fragments across the cells was a specific transcytotic event catalyzed by the pIgA-R, we performed an analogous internalization experiment with MDCK cells not transfected with pIgA-R. Non-transfected cells internalized low amount of ligand (<2%) and did not transport the [Fab]$_2$ fragments to the apical surface of the cells.

BFA was used at a concentration of 10 μM throughout the 37°C incubation period. For treatment with cholera toxin, MDCK cells were incubated at 37°C with cholera toxin for 1 h at 10 μg/ml, and with media for the next 4 h. Pertussis toxin was used at a concentration of 1 μg/ml throughout the 37°C incubation period.

Ricin-HRP internalization from the apical surface was performed as indicated for the pIgA-R ligand, except that 10 mM lactose washes were used to remove the bound ricin after the 17°C period. Incubation at 37°C was also performed in the presence of 10 mM lactose, to recover the ricin-HRP recycling back to the apical surface. HRP activity was tested using as a substrate Turbo-TMB (Pierce, Rockford, IL) and optical density was determined at 450 nm.

Fluorescence Confocal Microscopy

The in vivo transcytotic assay was used as previously described except that the ligand was labeled with fluorescent dyes, Cy3 or FITC, as described by the manufacturer. [Fab]$_2$ fragment-Cy3 or -FITC was added to the basolateral (10 μg/ml) or apical media (20 μg/ml) and the cells incubated for 4 h at 17°C. Cells were then washed and incubated at 37°C for different periods of time, under microtubule-depolymerizing conditions (as described earlier). Other markers, holo-dog transferrin-FITC (50 μg/ml), ricin-FITC (10 μg/ml), or BSA-FITC (5-6 mg/ml) were added simultaneously with pIgA-R ligand-Cy3 to either the basolateral or the apical media. Double-labeling fluorescence confocal microscopy was used to analyze the colocalization between their staining patterns, under different internalization conditions. The cells were washed on ice five times with PBS^{++} for 5 min, and then fixed for 10 min in 3% paraformaldehyde in PBS at room temperature. Cells were washed extensively with PBS, the filters removed and mounted in 50% glycerol in PBS. Confocal imaging was performed using an MRC 600 confocal microscope mounted on a optiphot II Nikkon with a 60× oil immersion lens BioRad Labs. (Hercules, CT). Differences between the photomultiplier tube sensitivity of the two different fluorochromes emission spectrum were compensated during the collection of the data to obtain images of equivalent brightness. Black and white photographs were obtained using a Screen-Star system and T-Max 400 films from Kodak. Color photographs were obtained using the same system but with EKTAR-100 film from Kodak.

Results

17°C Block Traps Transcytotic Cargo in an Apically Localized Compartment

To analyze the pathway traversed by basolaterally internalized transcytotic cargo after its sorting from the basolateral endosome and before its release into the apical medium, we used a 17°C temperature block which has been shown to allow transcytotic cargo sorting and its microtubule-dependent movement from the basolateral to the apical region of cells, but prevents its apical release. To examine the compartment in which transcytotic cargo accumulates during the 17°C block, Cy3-labeled pseudo-ligand was internalized at 17°C for 4 h from the basolateral surface of MDCK cells transfected with rabbit pIgA-R and grown on filter supports. MDCK cells were polarized and formed a tight monolayer as assayed by polarized transferrin internalization and by impermeability of the monolayer to WGA (data not shown). After internalization, the cells were washed, processed for fluorescence microscopy, and examined for distribution of ligand-Cy3 using a confocal microscope. 18 horizontal planes (1 μm apart) spanning the total height of the cells were obtained. The average height of the filter-grown cells was of 15–20 μm, which is within the range reported by Bacalao and Stelzer (1989) for a fully polarized cell monolayer. The pIgA-R ligand panels in Fig. 1 *A* show 9 of the original 18 focal planes, each ~2 μm apart, scanning the cells from the apical (1 μm from the apical PM) to the basolateral side (17 μm from the apical PM). pIgA-R ligand internalized into cells at 17°C is present in punctate structures distributed throughout the cell, but concentrated in the apical (−1 to −5

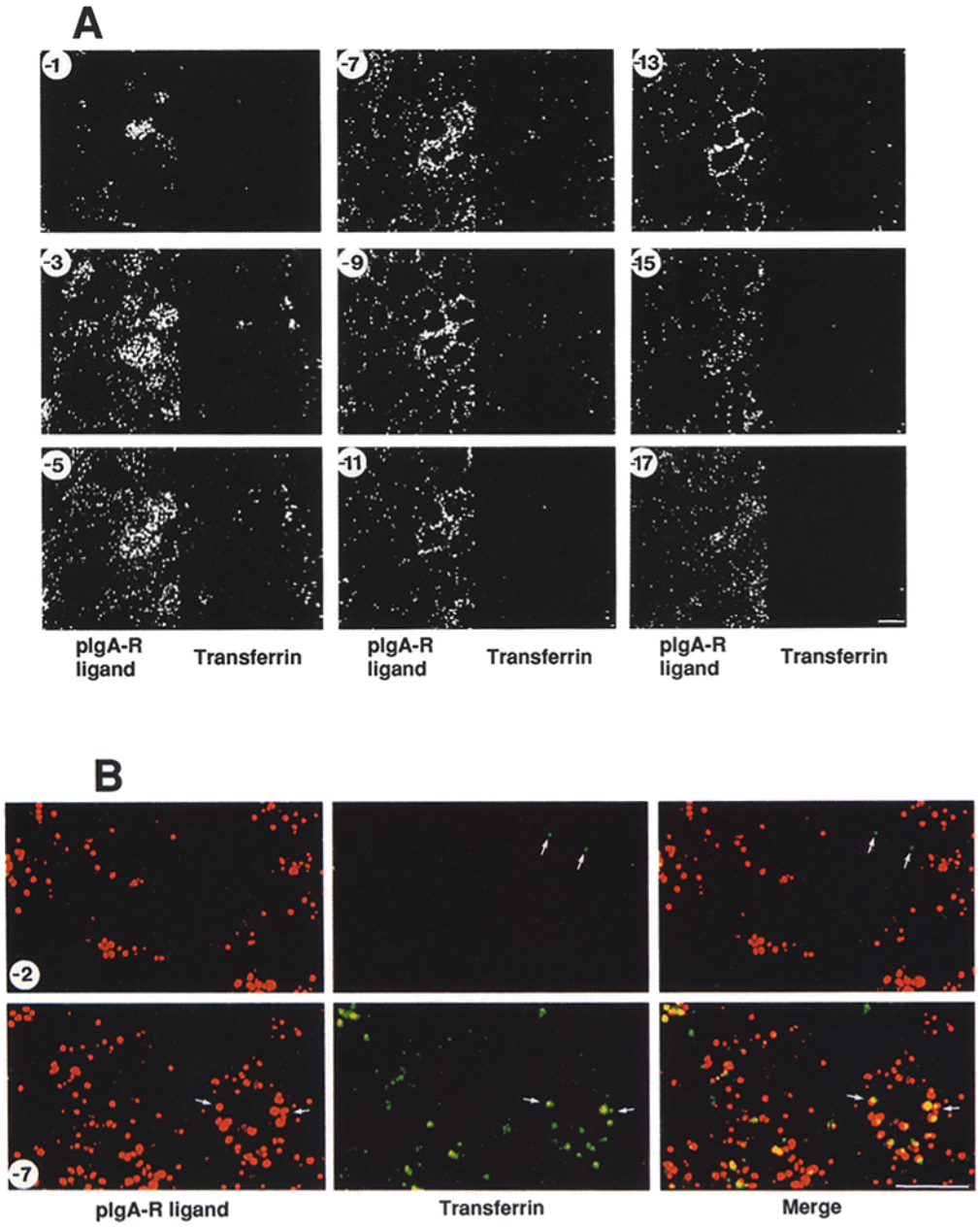


Figure 1. pIgA-R ligand and transferrin are in distinct compartments after the 17°C internalization period. (*A*) MDCK cells internalized pIgA-R ligand-Cy3 and transferrin-FITC for 4 h at 17°C from the basolateral surface. The cells were then processed for double-label fluorescence microscopy. Confocal microscope was used to scan the MDCK cell layer along its vertical axis, between the apical PM and the basolateral PM, generating 18 consecutive (1 μm apart) horizontal focal planes. 9 of the original 18 focal planes are shown representing the top/apical (−1 to −5 μm which correspond to 1–5 μm from the apical PM), the medial (−7 to −11 μm) and the lower/basolateral (−13 to −17 μm) regions of the cells. A double filter was used to scan simultaneously for the Cy3 (*pIgA-R ligand* panels) and FITC (*transferrin* panels) signals. Bar, 10 μm. (*B*) Enlargements of two focal planes are shown, representing the top/apical (−2 μm) and the medial (−7 μm) region of the cells. The yellow color in the merge panels (*arrows*) indicates overlap between the pIgA-R ligand-Cy3 (*red*) signal and the transferrin-FITC (*green*) signal. Bar, 10 μm.

μm) region of the cells and less common in the medial (−7 to −11 μm) and in the basolateral (−13 to −17 μm) regions of cells. The presence of ligand within the apical regions of the cells indicates that translocation from the basolateral to the apical region is not inhibited by the 17°C block (although it might be slowed) and is in agreement with the findings of Hunziker et al. (1990) showing that in cells loaded under the same conditions with dIgA, the majority of dIgA-containing structures accumulate in the upper part of the cell.

To examine whether ligand accumulated at 17°C is capable of reaching the apical PM, we compared the release of radiolabeled pIgA-R ligand into the apical and basolateral media after internalization either at 17°C for 4 h or at 37°C for 10 min and subsequent chase at 37°C. As shown in Fig. 2 *A*, ~48% of pIgA-R ligand internalized at 17°C is transcytosed and released into the apical media when the cells are incubated at 37°C for 5 h. During the 17°C internalization period, no ligand is released into the apical media (data not shown). The same amount of ligand is transcytosed when the internalization was performed at 37°C for 10 min but the release shows a slight lag time in comparison to the 17°C internalization. Basolateral recycling of ligand internalized at 17°C occurs faster than transcytosis, with close to 30% of the internalized pIgA-R ligand reaching the basolateral medium after 60 min chase at 37°C (Fig. 2 *B*). Similar recycling kinetics are observed when ligand internalized at 37°C is analyzed: slight increase in the overall recycling (29% at 1 h and 38% at 5 h) is observed. The slow rate of transcytosis observed in our system is probably due to technical reasons, most likely a result of prolonged washing on ice which we use to remove unbound ligand after the internalization period. We also need to stress that direct comparison between rates of transcytosis obtained in different studies is difficult since transcytosis has been studied using different types of

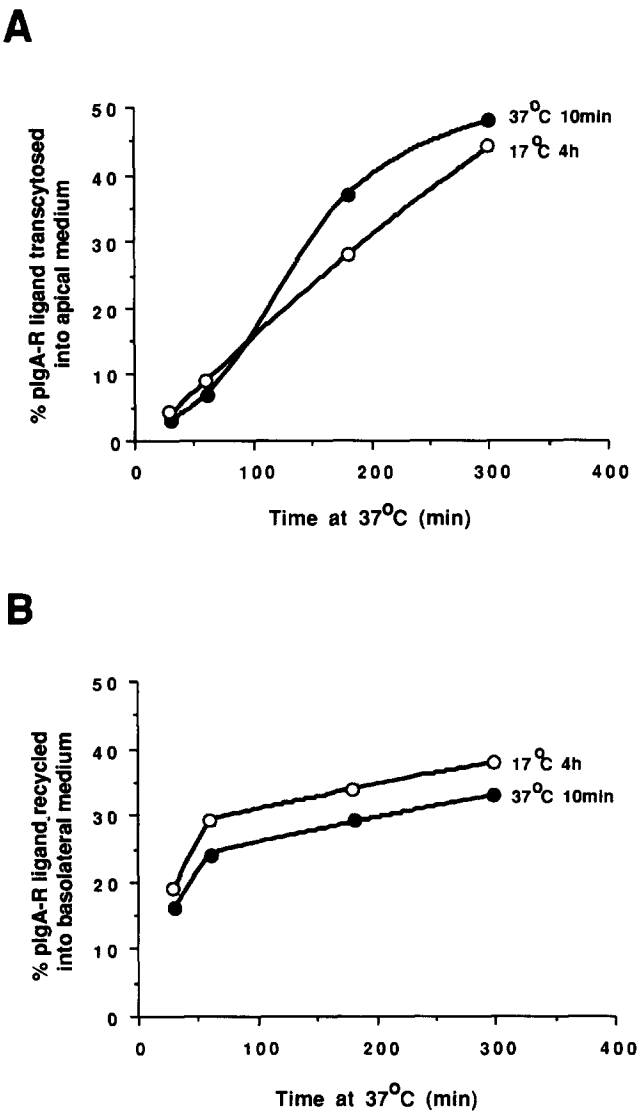


Figure 2. Quantitation of the transcytotic and recycling pathways of pIgA-R ligand. Radiolabeled pIgA-R ligand was internalized from the basolateral surface of MDCK cells for 4 h at 17°C (*dark circles*) or for 10 min at 37°C (*open circles*). Cells were then washed and transferred to 37°C for different periods of time. The apical and basolateral medium were collected and immunoprecipitated with anti-ligand antibodies. Radioactivity released into apical or basolateral medium was plotted as % of total label present in both media and in cells on filters. (*A*) Basolateral-to-apical transcytosis (ligand released into the apical media). (*B*) Basolateral recycling (ligand released into the basolateral media).

pIgA-R ligands i.e., dIgA, monomeric Fab or dimeric Fab and it appears that different ligands might be handled differently by MDCK cells (Hirt et al., 1993; Bomsel, M., personal communication).

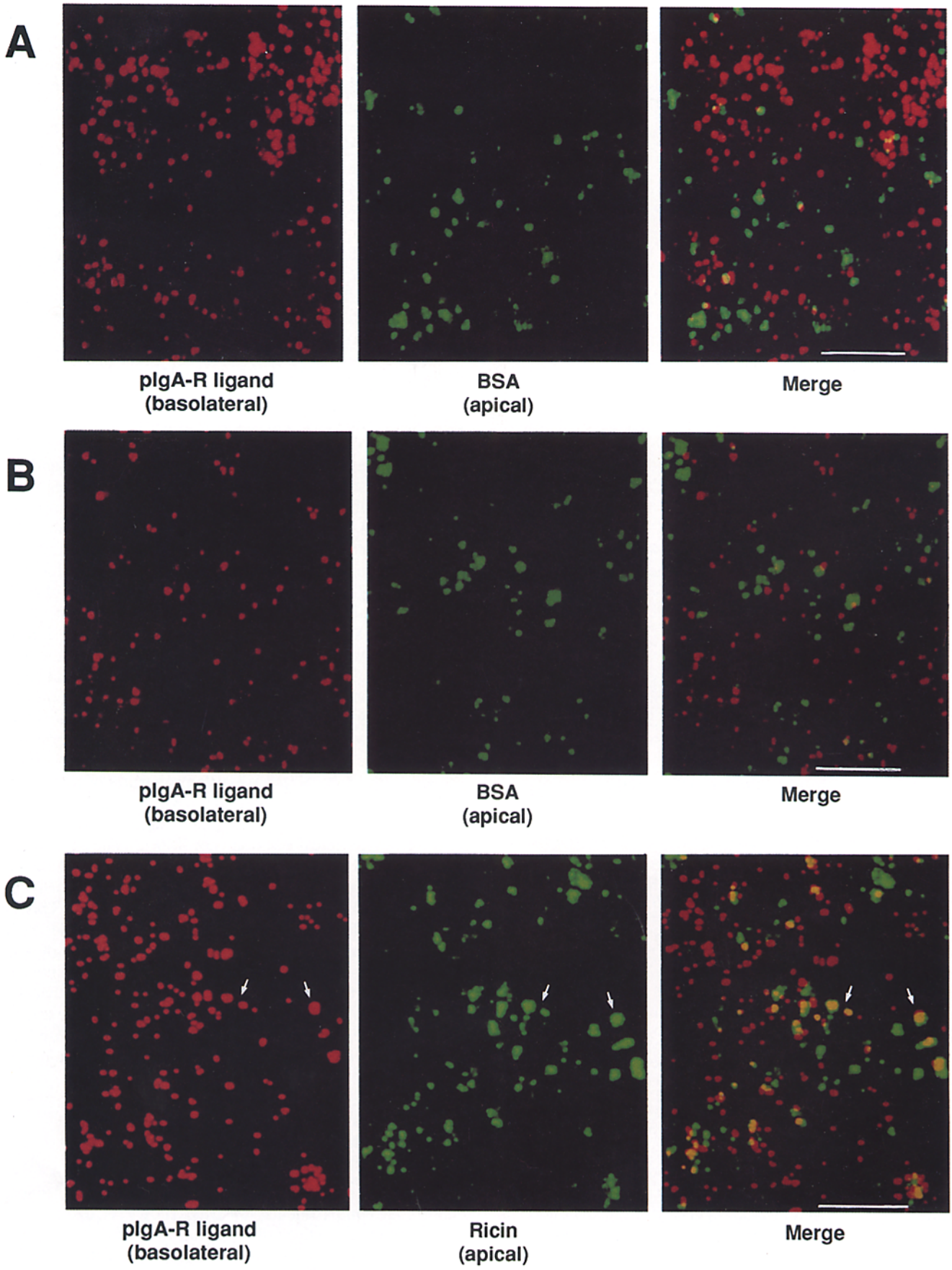
To ensure that the internalized ligand was not degraded, we measured the amount of label recovered during immunoprecipitation of ligand (intact ligand) from media and cells and compared it to the amount of label recovered in the supernatant after the immunoprecipitation (degraded ligand). Less than 5% of the label was recovered in the supernatant and might represent degradation products.

The Apical Transcytotic Compartment Is Not a Basolateral Endosome

Since the basolateral surface of MDCK cells extends up to ~5 μm from the apical PM (where tight junctions are localized when assayed by WGA apical binding at low temperature—data not shown), it was possible that the pIgA-R ligand trapped at 17°C was in the basolateral endosomes localized above and around the nucleus. We therefore determined the accessibility of the apical transcytotic compartment to transferrin, a protein shown to be endocytosed and recycled through the basolateral endosome in MDCK cells (Fuller and Simons, 1986; Podbilewicz and Mellman, 1990). pIgA-R ligand and canine holo-transferrin, labeled with different fluorochromes (Cy3 and FITC, respectively), were cointernalized from the basolateral surface of MDCK cells for 4 h at 17°C. The cells were then washed, processed for double-label fluorescent microscopy, and analyzed using a confocal microscope (Fig. 1 *A*, transferrin panels). Transferrin was mainly localized in the apical/medial (−3 to −9 μm) part of the cells, consistent with its published distribution within polarized cells (Hughson and Hopkins, 1990). To examine more closely the pIgA-R ligand containing- and the transferrin containing-compartments, all focal planes were examined at higher magnification and the level of colocalization analyzed by merging the Cy3 (pIgA-R ligand) and the FITC (transferrin) signals. Two representative focal planes from either the top/apical (−2 μm panel) or the medial (−7 μm panel) regions of the cells are shown in Fig. 1 *B*. pIgA-R ligand and transferrin can be detected in both regions of the cells but the amount of transferrin in the top/apical area is extremely low. More significantly, transferrin present within that region does not colocalize with the pIgA-R ligand (Fig. 1 *B*, −2 μm panel, *arrows*). Similarly, no colocalization was seen in planes taken between 1 and 4 μm from the apical PM (data not shown). Transferrin is more abundant in the medial portion of the cell (5 μm–11 μm from apical PM), and as shown in Fig. 1 *B*, −7 μm panel, a proportion of it colocalizes with the pIgA-R ligand (*arrows*). A similar level of colocalization was observed in planes taken between 5 and 11 μm from the apical PM (data not shown). The colocalization most likely reflects the presence of both pIgA-R ligand and transferrin in the basolateral endosomal compartment before they are sorted out into different pathways. This is consistent with the recent finding that an early endosomal fraction pepared from rat liver contains both the pIgA and the transferrin receptors (Casciola-Rosen and Hubbard, 1992). The data presented in Fig. 1, *A* and *B* show that the majority, if not all, of apical (within 4 μm from the apical PM) structures containing pIgA-R ligand internalized at 17°C are distinct from the transferrin containing basolateral endosome.

The Apical Transcytotic Compartment Is Not Accessible to Apically Internalized Fluid-Phase Marker

Since MDCK cells contain distinct basolateral and apical endosomal compartments, we used BSA-FITC as a fluid-phase marker to analyze the relationship between the apical transcytotic compartment and the apical endocytic pathway. pIgA-R ligand labeled with Cy3 was internalized from the basolateral surface of the cells for 4 h at 17°C. During the


A
pIgA-R ligand
(basolateral)
BSA
(apical)
Merge
B
pIgA-R ligand
(basolateral)
BSA
(apical)
Merge
C
pIgA-R ligand
(basolateral)
Ricin
(apical)
Merge

last 2 h of the internalization period, BSA-FITC was added to the apical surface of the same cells. As shown previously, fluid-phase endocytosis and recycling occurs at 17°C but delivery of internalized material to late compartments of the endosomal/lysosomal pathway is inhibited (Dunn et al., 1980; Bomsel et al., 1989). Consequently, BSA-FITC internalized at this reduced temperature will fill only the early endosomal compartment. After internalization, the cells were washed and processed for double-label fluorescence microscopy. The apical regions (−1 to −4 μm from the apical PM) of the cells were scanned by confocal microscopy to obtain four focal planes.

As shown in Fig. 3 *A*, both pIgA-R ligand-Cy3 and BSA-FITC are present in the top/apical region of the cells, in compartments similar in morphology and localized in close proximity. In general, BSA-FITC–containing structures are found in the periphery of the cells while pIgA-R ligand-Cy3 containing compartments accumulate in a more central region. The peripheral localization of the fluid-phase marker is consistent with its inability to reach late endocytic compartments at 17°C (Dunn et al., 1980; Bomsel et al., 1989). When the signals from the two fluorochromes were merged, the level of colocalization between the compartments was very low indicating that the transcytotic compartment does not colocalize with fluid phase markers present within the apical early endosome.

To examine the relationship between the transcytotic compartment and the late endosomal and lysosomal systems, we compared the localization of the transcytotic compartment (marked by pIgA-R ligand internalized for 4 h at 17°C) to that of late endosomes and lysosomes (containing BSA internalized during subsequent chase for 1 h at 37°C). The fluid phase BSA was internalized from the apical surface since it has been shown that cargo endocytosed for more than 30 min at 37°C from either surface of MDCK cells enters a shared late endosomal and lysosomal system and thus the internalized BSA will label the entire cellular late endosomal-lysosomal system (Bomsel et al., 1989; Parton et al., 1989). As shown in Fig. 3 *B*, no colocalization was observed, indicating that the transcytotic compartment is distinct from late endosomes or lysosomes.

The Apical Transcytotic Compartment Is Accessible to Apically Internalized Ricin

It has been shown previously that fluid-phase markers are sorted from cointernalized membrane-bound markers within the early endosome (Maxfield and Yamashiro, 1991). Since pIgA-R ligand remains bound to its receptor during transcytosis it is, in effect, a membrane-bound marker. Thus, we posited that pIgA-R ligand might be present within an apical endosomal compartment containing membrane-bound markers sorted away from fluid-phase material. To examine the relationship between the apical transcytotic compartment and the apical endosomal compartment involved in membrane traffic, we used ricin, a 60-kD lectin which binds to galactose residues of membrane glycoproteins and glycolipids and thereby acts as a non-specific probe for membrane flow. Ricin can be used as a general marker for endocytosis and recycling of apically internalized membrane proteins since van Deurs and co-workers (van Deurs et al., 1990; Prydz et al., 1992) have previously demonstrated that the majority of apically internalized ricin recycles back to the apical PM, while a small percentage is transcytosed to the basolateral PM or transported to the lysosomes and the Golgi complex.

MDCK cells internalized pIgA-R ligand-Cy3 from the basolateral surface for 4 h at 17°C. During the last 2 h, ricin-FITC was internalized from the apical surface of the cells. The cells were processed for double-label fluorescence microscopy and the apical (1–4 μm from the apical PM) region of the cells was then analyzed using a confocal microscope. A representative focal plane is presented. As shown in Fig. 3 *C*, both pIgA-R ligand and ricin are detected in the apical region of the cells in morphologically similar structures. A merge of the Cy3 and FITC signals results in marked colocalization between the ricin-FITC and pIgA-R ligand-Cy3 containing compartments (*arrows*). Although the colocalization is not complete, it is significantly higher than the colocalization of BSA-FITC and pIgA-R ligand-Cy3 internalized under the same conditions (compare Fig. 2, *A* and *B*, merge panels). These results indicate that the apical transcytotic compartment overlaps with an apical membrane-recycling compartment and that *apically* internalized fluid-phase and membrane-bound markers segregate within the apical endosomal system.

The Apical Transcytotic Compartment Is Accessible to Apically Internalized pIgA-R Ligand

pIgA-R ligand is internalized from both the basolateral and the apical PM domains in polarized MDCK cells transfected with pIgA-R (Breitfeld et al., 1989). However, after endocytosis, the ligand follows distinct transport pathways: while basolaterally internalized pIgA-R ligand is predominantly transcytosed, the majority of apically endocytosed pIgA-R ligand recycles back to the apical PM (Breitfeld et al., 1989). Therefore, pIgA-R ligand internalized from the apical surface of MDCK cells can be used as a specific marker for the apical receptor-mediated recycling pathway. To examine the relationship between the transcytotic and the apical pIgA-R recycling compartments, MDCK cells internalized pIgA-R ligand-Cy3 basolaterally and pIgA-R ligand-FITC apically for 3 h at 17°C. The cells were then washed and processed for double-label fluorescence microscopy. Four focal planes (1, 2, 3, and 4 μm from the apical surface) were obtained and are shown in Fig. 4. Basolaterally internalized pIgA-R ligand is detected predominantly 3 and 4 μm below the apical PM

Figure 3. The apical transcytotic compartment is selectively accessible to apically added markers. pIgA-R ligand-Cy3 was internalized for 4 h at 17°C from the basolateral surface of the MDCK cells. BSA-FITC was added to the apical surface of the cells during the last 2 h of the internalization period (*A*) or during 1 h at 37°C chase after the internalization period (*B*). In *C*, ricin-FITC was added to the apical surface of the cells during the last 2 h of the internalization period. The cells were then processed for double-label fluorescence microscopy. Confocal microscope was used to scan the top/apical (1–4 μm from the apical PM) regions of the cells and a representative focal plane is shown. Merge panels were produced by overlapping the Cy3 and the FITC signals. Bar, 10 μm.

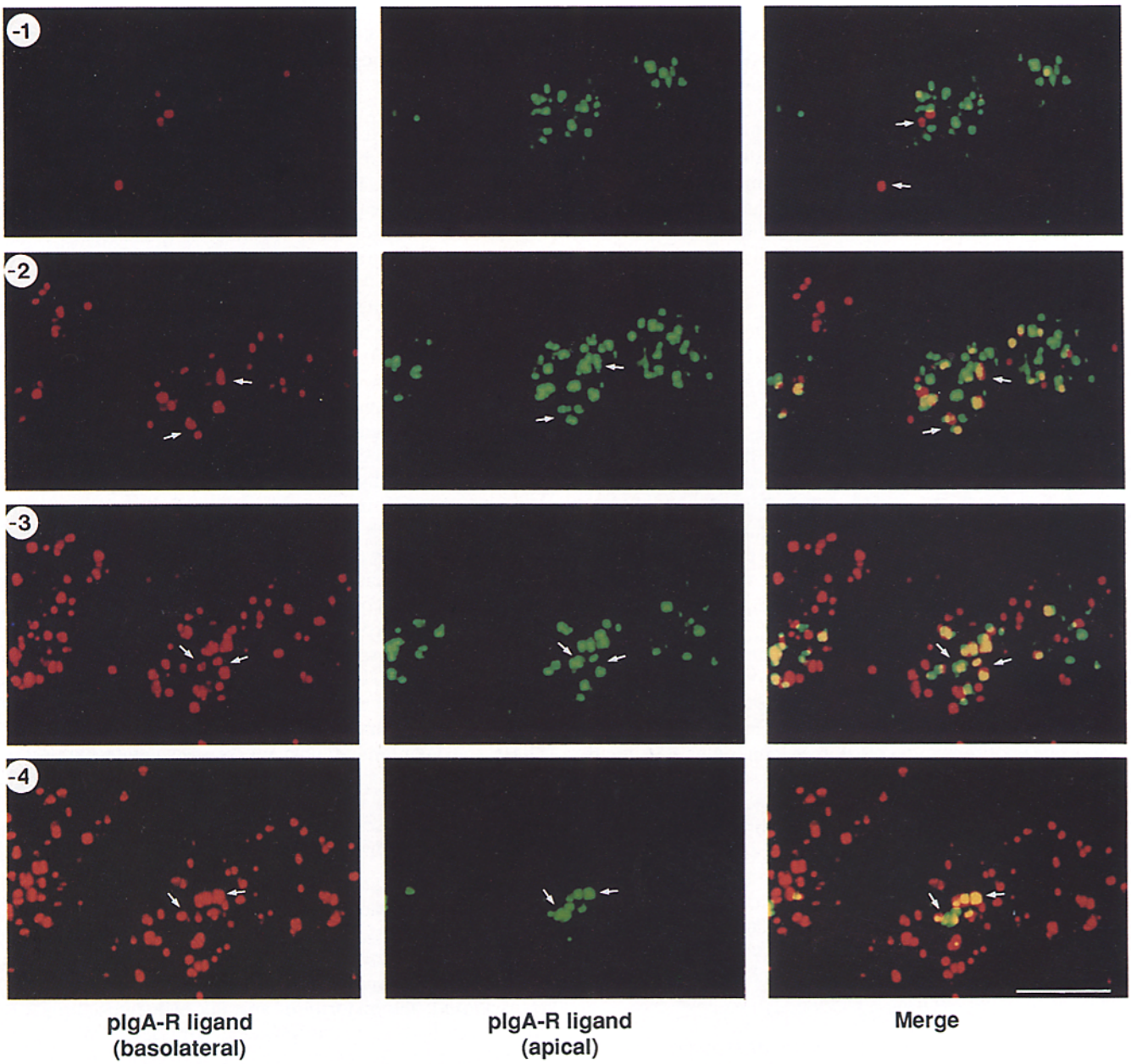


Figure 4. Basolaterally and apically internalized pIgA-R ligand colocalize in an apical compartment. MDCK cells internalized pIgA-R ligand-Cy3 (added to the basolateral surface) and pIgA-R ligand-FITC (added to the apical surface) for 3 h at 17°C. The cells were then processed for double-label fluorescence microscopy. Confocal microscope was used to scan the top/apical (1–4 μm from the apical PM) regions of the cells and the four focal planes obtained are shown. Merge panels were produced by overlapping the Cy3 and the FITC signals. Bar, 10 μm.

while apically internalized pIgA-R ligand is concentrated in more apical regions. Merging the Cy3 and the FITC signals shows significant colocalization of the two markers (*arrows*) in all except the most apical (−1 μm) focal plane (*arrows*). It appears, therefore, that the apical transcytotic compartment and the apical receptor-mediated recycling compartment overlap within the apical region of the cells.

Apically Internalized Fluid-Phase Marker and Apically Internalized pIgA-R Ligand Are Present in Distinct Apical Endosomal Compartments

We have shown above that pIgA-R ligand internalized from the basolateral surface at 17°C is delivered to an apical transcytotic compartment which is not accessible to fluid-phase marker internalized from the apical PM but which receives apically endocytosed membrane-associated markers. These results suggest that the apical endosomal system is divided into distinct compartments, one handling fluid phase cargo and another involved in membrane traffic. To test this hypothesis, pIgA-R ligand-Cy3 and BSA-FITC were cointernalized from the apical surface of MDCK cells at 17°C for 2 h. The cells were then processed as above and the apical region of cells examined by confocal microscopy. As shown in Fig. 5 *A*, both pIgA-R ligand and BSA are present within the apical region of the cells but the compartments

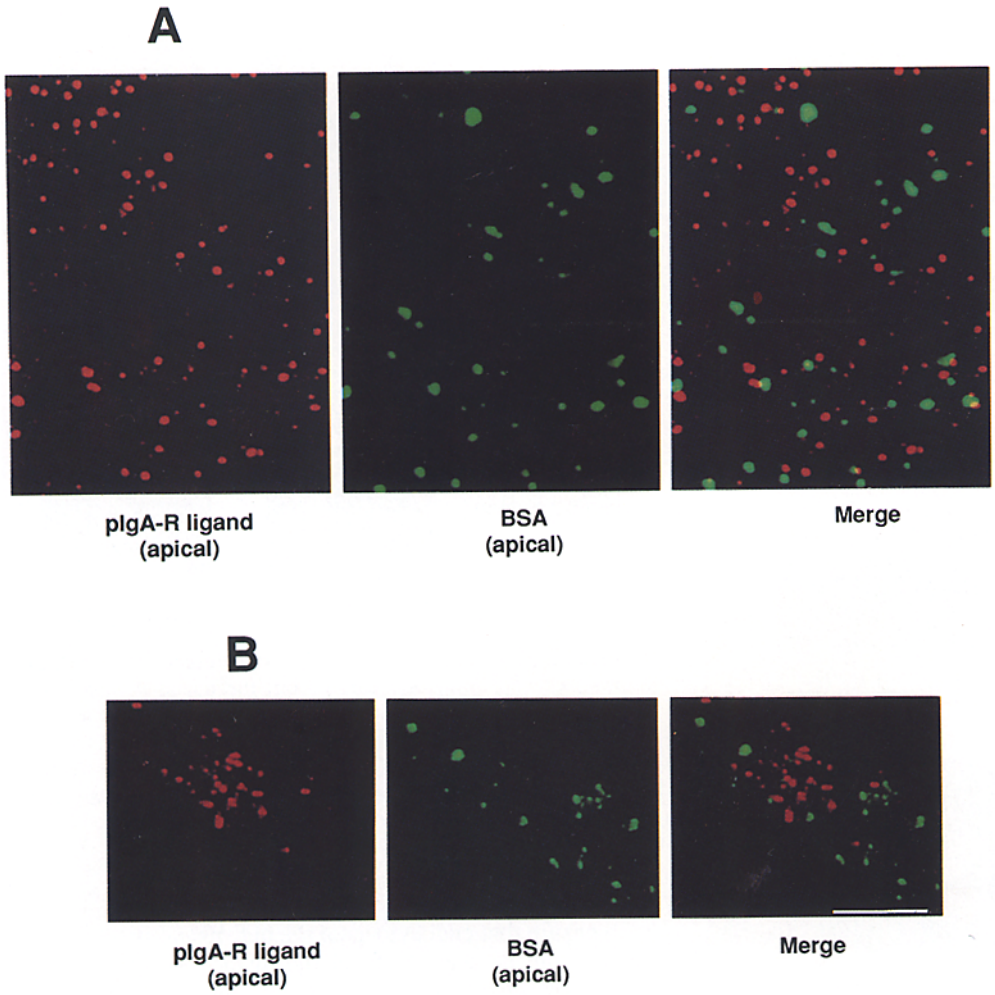


Figure 5. pIgA-R ligand and a fluid-phase marker cointernalized from the apical surface are present in distinct apical compartments. pIgA-R ligand-Cy3 and BSA-FITC were added to the apical surface of MDCK cells and internalized for 2 h at 17°C (*A*) or for 10 min at 37°C (*B*). The cells were then processed for double-label fluorescence microscopy. Confocal microscope was used to scan the top/apical region (1–4 μm from the apical PM) of the cells and a representative focal plane is shown. Merge panels were produced by overlapping the Cy3 and the FITC signals. Bar, 10 μm.

containing the two ligands, although in close proximity, do not show significant colocalization. The results suggest that the separation of markers occurs within the early endosome since the internalization is carried out at 17°C, the temperature at which endocytosed proteins should be prevented from moving into late endosomes. However, to ensure that the separation is not due to BSA-FITC partially bypassing the temperature block and gaining entrance into the late endosomes, we repeated the experiment at 37°C. pIgA-R ligand-Cy3 and BSA-FITC were internalized at 37°C for 10 min, a time period sufficient for BSA-FITC to fill the early endosomes but not the late endosomes (defined as a compartment containing mannose 6-P receptor) (Bomsel et al., 1989). As shown in Fig. 5 *B*, pIgA-R ligand and BSA were present in neighboring compartments but a merge of the Cy3 and FITC signals did not show colocalization. The data suggest that markers internalized from the apical surface are rapidly sorted within the apical early endosome and are restricted to either the fluid-phase or the membrane recycling compartments. This segregation phenomenon has been observed in electron microscopic analyses of receptor-ligand dissociation after endocytosis in nonpolarized cells or uptake from the basolateral PM (Geuze et al., 1984; Mueller and Hubbard, 1986; Dunn et al., 1989). It appears that the apical endosome behaves the same way and effectively segregates markers into specific sub-domains.

Entry into the Apical Transcytotic Compartment Does Not Involve Recycling from the Apical PM

The data presented above suggest that the basolateral to apical transcytotic pathway includes movement of transcytotic cargo through an apical endosome before the cargo is delivered to the apical PM. However, a major concern in interpreting such studies is to distinguish between a situation in which transcytotic cargo enters the apical recycling endosome before being delivered to the apical PM from one where pIgA-R ligand first moves to the apical PM and is then reendocytosed from the apical surface and delivered to the apical early endosome. To examine whether entry into the apical endosome occurs directly or via reendocytosis, we added Cy3 labeled pIgA-R ligand basolaterally and FITC-labeled anti-ligand ($[Fab]_2$ fragment of rabbit IgG raised against goat IgG) apically and incubated the cells for various times and at different temperatures. Anti-ligand-FITC will be internalized only if it can bind to ligand exposed on the apical PM and be endocytosed along with it; we have previously determined that anti-ligand is not internalized when added to the apical surface of cells in the absence of ligand (data not shown). As shown in Fig. 6 *A*, when the incubation with basolaterally added ligand and apically added anti-ligand was done at 17°C for 4 h, no significant FITC staining

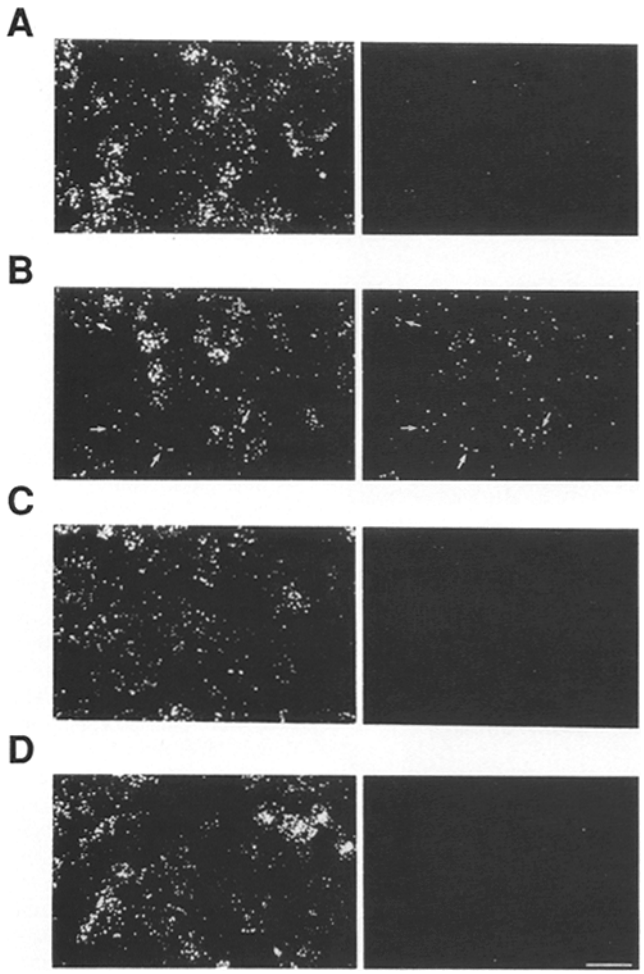


Figure 6. Entry of pIgA-R ligand into the apical transcytotic compartment does not involve reendocytosis from the apical surface. pIgA-R ligand-Cy3 (10 μg/ml) was added to the basolateral surface and anti-ligand-FITC (20 μg/ml) was added to the apical surface of MDCK cells. The cells were incubated either for 4 h at 17°C (*A*) or for 2 h at 37°C (*B*). Alternatively, pIgA-R ligand-Cy3 was added to the apical surface and anti-ligand-FITC to the basolateral surface of MDCK cells and the cells incubated either for 4 h at 17°C (*C*) or for 2 h at 37°C (*D*). The cells were processed for double-label fluorescence microscopy. Confocal microscope was used to scan the top/apical region (1–4 μm for the apical PM) of the cells and a representative focal plane is shown. Each panel consists of a double image showing the ligand-Cy3 staining (*left panel*) and the anti-ligand-FITC staining (*right panel*). Bar, 10 μm.

was detected inside the cells suggesting that the ligand does not reach the apical PM.

To show that the ligand and anti-ligand can interact with each other, we incubated cells with basolaterally added ligand and apically added anti-ligand for 2 h at 37°C. As shown previously in Fig. 2 *A*, ~20% of transcytotic ligand reaches the apical PM after a 2-h 37°C incubation. Since apical recycling of ligand has been shown to occur (Breitfeld et al., 1989; Hunziker et al., 1990), we expected that the anti-ligand would interact with the ligand at the apical PM and be endocytosed together. As shown in Fig. 6 *B*, significant amounts of anti-ligand are present within the apical region of the cells and show complete colocalization with the ligand (*arrows*).

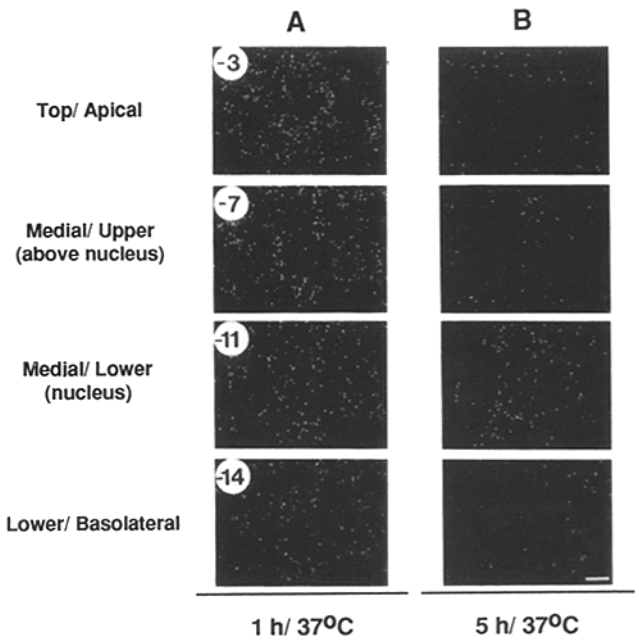


Figure 7. Incubation of cells at 37°C in the presence of nocodazole allows exit of transcytotic cargo from the apical compartment. MDCK cells internalized pIgA-R ligand-Cy3 from the basolateral surface for 4 h at 17°C. The cells were then supplemented with nocodazole and shifted to 37°C for 1 (*A*) or 5 h (*B*). The cells were processed for confocal fluorescence microscopy as described in the legend to Fig. 1. Bar, 10 μm.

To ensure that endocytosis of the anti-ligand is totally dependent on the availability of the ligand, we repeated the above experiments but added the anti-ligand to the basolateral side of MDCK cells and the ligand to the apical side. Since pIgA-R ligand added to the apical side recycles only to the apical surface, it should never be exposed to the anti-ligand added to the basolateral media. As shown in Fig. 6, *C* and *D*, after incubation at either 17 or 37°C, respectively, no FITC staining was detected inside the cells. These data indicate that pIgA-R ligand internalized from the basolateral surface at 17°C enters the apical endosome without prior delivery to the apical PM.

Analysis of Transcytotic Ligand Movement from the Apical Compartment to the Apical Medium

To examine only the late steps of transcytosis, we dissected the overall pathway into the late stages (exit of transcytotic cargo from the apical transcytotic compartment and its delivery to the apical PM) and the early stages (delivery into the apical transcytotic compartment). We preloaded the apical compartment with ligand internalized from the basolateral surface at 17°C, and then chased the ligand from that compartment by shifting the cells to 37°C in the presence of a microtubule-disrupting drug, nocodazole. In the presence of nocodazole, transcytotic cargo within the apical transcytotic compartment (this cargo is already past the microtubule-requiring step) will be able to move to the apical PM (this step does not require microtubules; Hunziker et al., 1990; Breitfeld et al., 1990) but cargo present in proximal compartments will not enter the apical transcytotic compartment

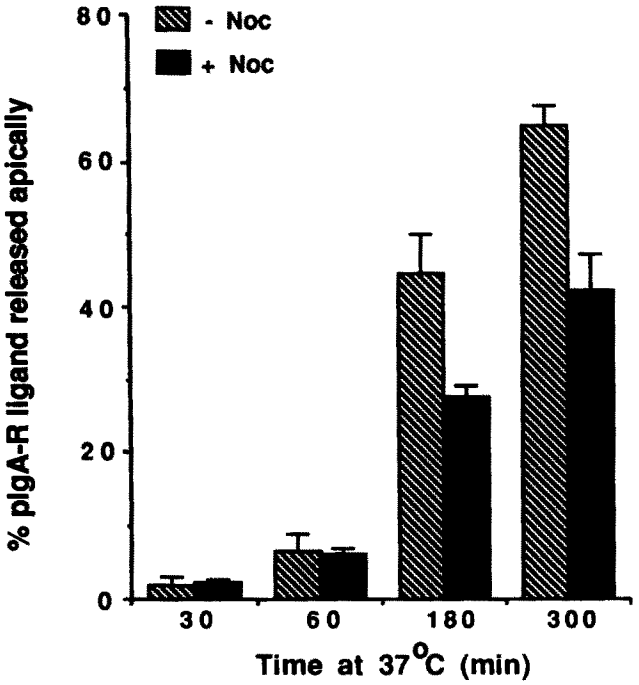


Figure 8. Effect of microtubule disruption on the basolateral-to-apical transcytosis of pIgA-R ligand. Radiolabeled pIgA-R ligand was internalized from the basolateral surface of MDCK cells for 4 h at 17°C. The cells were then shifted to 37°C and incubated for different periods of time in the absence or presence of nocodazole. Percent values represent the amount of radiolabeled pIgA-R ligand released into the apical media, considering the total amount of pIgA-R ligand internalized into the transcytotic pathway as 100%. The bars represent the mean ± SD from 3–4 experiments.

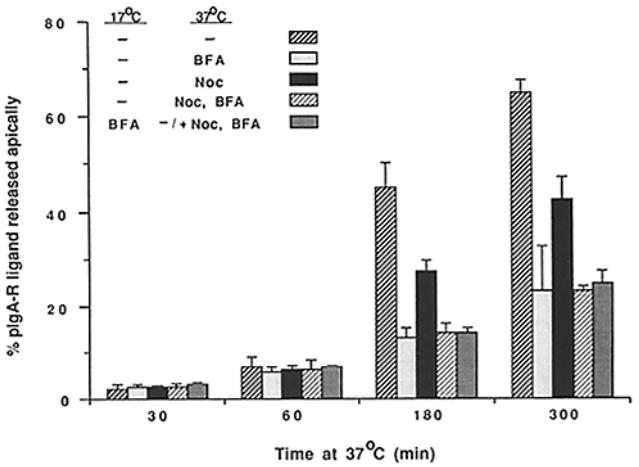


Figure 9. BFA inhibits post microtubule-dependent step of transcytotic pathway. Radiolabeled pIgA-R ligand was internalized from the basolateral surface of MDCK cells for 4 h at 17°C in the absence or presence of BFA. The cells were then shifted to 37°C for different periods of time in the presence or absence of BFA and/or nocodazole. Percent values represent the amount of radiolabeled pIgA-R ligand released into the apical media, considering the total amount of pIgA-R ligand internalized into the transcytotic pathway as 100%. The bars represent the mean ± SD from 2–3 experiments.

(since movement into this compartment requires microtubules), and will be recycled back to the basolateral surface.

To analyze the traffic of the pIgA-R ligand from the transcytotic apical compartment to the cell surface, we first incubated the cells with ligand for 4 h at 17°C to load the apical transcytotic compartment and then shifted the cells to 37°C in the presence of nocodazole. The cells were incubated at 37°C for 1 or 5 h and then processed for fluorescent microscopy: 16 focal planes (1 μm apart) were obtained, four of which are presented in Fig. 7.

After a 1-h chase at 37°C, in the presence of nocodazole at a time when minimal pIgA-R ligand is released into the apical medium (see below), pIgA-R ligand is present in the apical and medial parts of the cells but is slightly less abundant in the basolateral region (Fig. 7 *A*). The pattern is similar to that seen when distribution of pIgA-R ligand is examined after the 17°C internalization without chase (see Fig. 1, pIgA-R ligand panels), except that the basolateral region contains less pIgA-R ligand, probably due to its recycling into the basolateral medium. When cells preloaded at 17°C are incubated at 37°C for 5 h, in the presence of nocodazole, a substantial reduction in the intensity of the pIgA-R ligand signal in the apical and medial/upper regions of the cells is observed (Fig. 7 *B*), suggesting that the pIgA-R ligand is being released into the apical medium (see below). At 1 and 5 h of nocodazole treatment, microtubule organization was analyzed by immunofluorescence with anti-tubulin antibodies: only diffuse cytosolic staining was observed (data not shown) indicating that the release of ligand occurs from compartments not dependent on microtubule-powered traffic to deliver their cargo to the cell surface. A substantial proportion of pIgA-R ligand-containing structures remains in the medial/lower regions of the cells after a 5-h chase (compare the focal planes −11 in *A* and *B*). This is probably due to their inability to move into the apical region or to move back toward the basolateral PM and enter the basolateral recycling pathway.

The biochemical quantitation of pIgA-R ligand delivery to the apical medium under the conditions described above is shown in Fig. 8. In this and subsequent analyses, the amount of ligand released into the apical medium during the chase is represented as the % of the total ligand internalized into the transcytotic pathway. The 100% value is calculated as follows: medium is replaced after 30, 60, and 180 min. At the end of the 300-min chase at 37°C, radioactivity in all fractions is determined. The ligand remaining in the cells after 5 h is also counted and the two values are added together to represent the total ligand within the transcytotic pathway. The amount of label recovered in the apical medium at each time point is represented as a percentage of that value.

In the absence of nocodazole, ~65% of the pIgA-R ligand internalized into the transcytotic pathway during the 4 h at 17°C incubation, can subsequently be released apically when cells are shifted to 37°C for 5 h. In contrast, when the microtubular network is disrupted by nocodazole treatment, only ~42% of the pIgA-R ligand internalized into the transcytotic pathway is released into the apical media. This 42% represents the pIgA-R ligand present within apical compartments distal to the microtubule-dependent step. When nocodazole is added during the 17°C internalization and maintained throughout the chase, no pIgA-R ligand is released into the apical medium (data not shown).

BFA Inhibits Late Steps of Transcytosis

BFA has been shown to block an early step of the transcytotic pathway, presumably by inhibiting a critical sorting event necessary for pIgA-R-dIgA complex exit from the basolateral early endosome (Hunziker et al., 1991). Since the apical transcytotic compartment overlaps with the apical sorting endosome, we investigated the influence of BFA on the exit of pIgA-R ligand from the apical endosome.

MDCK cells were preloaded with pIgA-R ligand for 4 h at 17°C, and then shifted to 37°C in the presence or absence of nocodazole and BFA. As shown in Fig. 9 (and previously in Fig. 8), a 5-h chase at 37°C in the absence of nocodazole and BFA results in the apical release of ~65% of internalized pIgA-R ligand. Addition of BFA during the 37°C chase markedly (~70% reduction) inhibited the apical release of the pIgA-R ligand. To examine whether the BFA effect was due to the inhibition of cargo exit from the apical transcytotic compartment (in which case, BFA effect would still be observed when chase was performed in the presence of nocodazole) or to events before cargo entry into the apical transcytotic compartment (in which case, the level of apical release of cargo would be the same in the presence of nocodazole and presence or absence of BFA), we analyzed the BFA effect during chase in the presence of nocodazole. As shown in Fig. 9 (and previously in Fig. 8), ~40% of the pIgA-R ligand is released into the apical medium when a 5-h chase at 37°C is performed in the presence of nocodazole. When both nocodazole and BFA are added during the chase, a marked reduction in apically released pIgA-R ligand is seen. The amount (~20% of pIgA-R ligand internalized into the transcytotic pathway) of apically released pIgA-R ligand is identical to the amount released when BFA alone (without nocodazole) is present during the chase, suggesting that BFA inhibits a postmicrotubule requiring step of the transcytotic pathway. The amount of pIgA-R ligand released during a 5-h chase at 37°C in the presence of BFA (in the presence or absence of nocodazole) into the apical media is identical to the amount of pIgA-R ligand released when BFA is added during the 17°C incubation and maintained during the 37°C chase (in the presence or absence of nocodazole). Since BFA added during the 17°C internalization inhibits the entire transcytotic pathway by preventing exit of pIgA-R ligand from the basolateral endosome (Hunziker et al., 1991), these results suggest that the value of ~20% pIgA-R ligand seen released into the apical medium in the presence of BFA represents the background of our system.

To examine whether BFA inhibits exit of transcytotic cargo from the apical compartment or whether the cargo exits the compartment but instead of reaching the apical PM is detoured to some "dead-end" compartment, we analyzed the level of colocalization between basolaterally internalized pIgA-R ligand and apically internalized pIgA-R ligand after a 37°C chase in the presence of the drug. pIgA-R ligand-Cy3 and pIgA-R ligand-FITC were internalized for 4 h at 17°C from the basolateral or the apical surface of the cells, respectively. The cells were then supplemented with nocodazole and BFA and shifted to 37°C for 1 or 3 h. The cells were processed for double-label fluorescence microscopy and the apical regions of the cells (1–4 μm from the apical PM) were examined using a confocal microscope. As shown in 10 *A*, after a 1-h chase at 37°C, the basolaterally and apically internalized pIgA-R ligands colocalize within the apical transcytotic/endosomal compartment (*arrows*). The level of colocalization is analogous to that observed when apical regions of cells were examined after basolateral and apical internalization of pIgA-R ligand for 3 h at 17°C (see Fig. 4). When cells were examined after a 3-h chase at 37°C (Fig. 10 *B*), there was a decrease in the amount of pIgA-R ligand-FITC (ligand internalized from the apical surface) due to uninterrupted recycling in the presence of BFA (see below). However, the apically internalized pIgA-R ligand present in the cells colocalized with the transcytotic pIgA-R ligand (*arrows*). These results suggest that BFA does not perturb the apical recycling pathway but inhibits the transcytotic pathway by preventing transcytotic cargo from leaving the apical endosome.

Cholera Toxin (Gsα Activator) Inhibits Late Steps of Transcytosis

Since BFA has been shown to modulate the molecular pathway(s) connecting trimeric G proteins with their effectors (Ktistakis et al., 1992), we analyzed whether signal transducing trimeric G proteins are involved in the late stages of the transcytotic pathway.

Cholera toxin catalyzes the ADP-ribosylation of the α-subunits of the stimulatory G proteins, categorized by their ability to stimulate adenylate cyclase (Gill and Woolkalis, 1991). ADP-ribosylated α subunits have reduced GTPase activity and decreased affinity for $\beta\gamma$ subunits and are able to persistently activate their downstream effectors (Kaziro et al., 1991). To examine if Gs is involved in the traffic of the pIgA-R ligand from the apical transcytotic compartment to the apical PM, MDCK cells were preloaded with pIgA-R ligand at 17°C for 4 h, then supplemented with nocodazole, and shifted to 37°C in the presence of cholera toxin. A concentration of cholera toxin (10 μg/ml) analogous to amount routinely employed in studies of live cells (Fishman and Atikkan, 1980; Watkins et al., 1981) was used. As shown in Fig. 11 (and previously in Figs. 8 and 9), ~40% of the pIgA-R ligand present within the transcytotic pathway is apically released during a 5-h chase at 37°C in the presence of nocodazole. A significant decrease (down to ~25%) in the amount of pIgA-R ligand released into the apical media is observed when cholera toxin is added during the chase period, suggesting that activation of a Gs protein(s) inhibits the delivery of the pIgA-R ligand into the apical media. We examined the localization of transcytotic cargo after a 3-h chase in the presence of cholera toxin; using double-label confocal microscopy we observed colocalization of the pIgA-R ligands analogous to that seen in Fig. 10 *B* (data not shown). These results suggest that, like BFA, cholera toxin inhibits the exit of transcytotic cargo from the apical transcytotic/endosomal compartment.

To examine if Gs was the only G protein acting during the late steps of the transcytotic pathway, or if additional G proteins were involved, we tested the effect of pertussis toxin in our in vivo assay. It has been previously shown that passage of secretory proteins through the Golgi can be modulated by a pertussis toxin sensitive Gi (Stow et al., 1991). In our transcytotic system, addition of pertussis toxin (which ADP-ribosylates Gi and Go proteins and uncouples them from their receptors) to cells during the 37°C chase period or even before the in vivo assay (overnight treatment; data not shown)

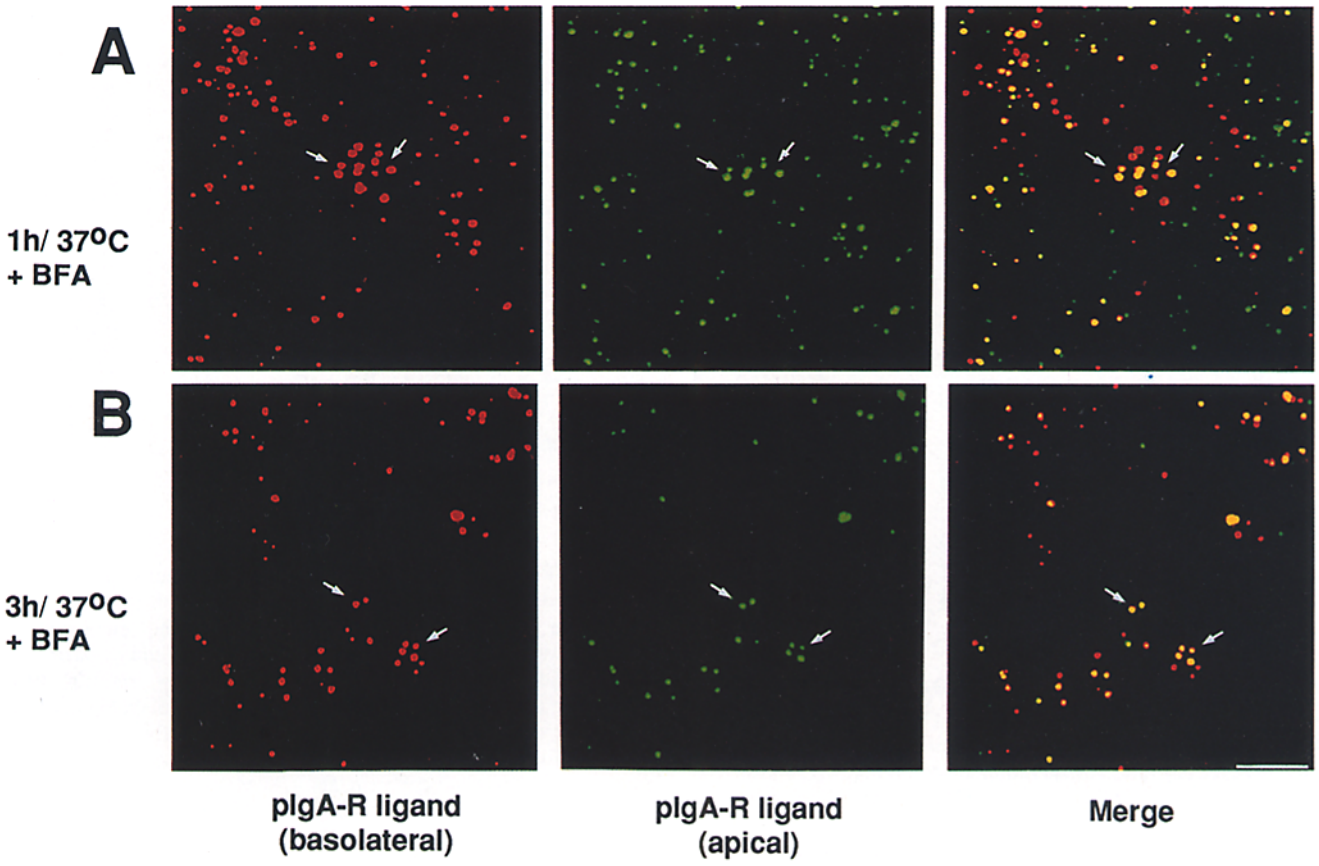


Figure 10. BFA prevents exit of transcytotic cargo from the apical transcytotic compartment. MDCK cells internalized pIgA-R ligand-Cy3 from the basolateral surface and pIgA-R ligand-FITC from the apical surface for 4 h at 17°C. The cells were then supplemented with nocodazole and BFA and incubated at 37°C for 1 (*A*) or 3 h (*B*). The cells were processed for double-label fluorescence microscopy. Confocal microscope was used to scan the top/apical (1–4 μm from the apical PM) regions of the cells and a representative focal plane is shown. Merge panels were produced by overlapping the Cy3 and the FITC signals. Bar, 10 μm.

had minimal effect on the delivery of the pIgA-R ligand into the apical media (Fig. 11). It appears therefore, that receptor-mediated activation of a Gi is not required for the completion of the transcytotic pathway. As expected, pertussis toxin treatment does not counteract the inhibitory effect of cholera toxin on the release of pIgA-R ligand into the apical media (data not shown).

BFA and Cholera Toxin Do Not Inhibit the Recycling of Apically Internalized pIgA-R Ligand or Ricin

Since BFA and cholera toxin inhibit the delivery of transcytotic cargo to the apical PM, we examined whether their action was specific, i.e., restricted to transcytotic traffic, or whether they inhibited all membrane traffic through the apical endosome. To do so, we analyzed the effect of BFA and cholera toxin on the recycling of pIgA-R ligand or ricin-HRP internalized from the apical surface.

pIgA-R ligand was internalized from the apical surface of MDCK cells for 2 h at 17°C, the cells were then treated with nocodazole and shifted to 37°C for various periods of time in the presence or absence of cholera toxin or BFA. As shown in Fig. 12 *A*, ~60% of internalized pIgA-R ligand is recycled to the apical surface in a 5-h chase at 37°C in the absence of BFA or cholera toxin. When BFA or cholera toxin are added during the 37°C chase, there is no significant effect (~57% of internalized pIgA-R ligand is recycled) on the apical release. The level of pIgA-R ligand recycled after apical internalization (~60%) is similar to that reported previously (Breitfeld et al., 1989).

Ricin-HRP was internalized from the apical surface for 2 h at 17°C, the cells were then treated with nocodazole and shifted to 37°C in the absence or presence of cholera toxin or BFA. As shown in Fig. 12 *B*, neither cholera toxin nor BFA significantly affected the apical recycling of ricin-HRP; as reported previously for untreated cells (Prydz et al., 1992) ~80% of internalized ricin-HRP was recycled to the apical surface. The minimal effect of BFA on apical recycling is in agreement with previous results (Prydz et al., 1992).

The data suggest that recycling from the apical endosome is not affected by BFA and cholera toxin but that these compounds specifically inhibit the completion of the transcytotic pathway.

Discussion

We have used pIgA-R ligand as a basolateral to apical transcytotic marker to study the late steps of the transcytotic pathway in polarized MDCK cells. Our assay is based on earlier findings by Hunziker et al. (1990), who showed that basolateral internalization of pIgA-R ligand by MDCK cells grown

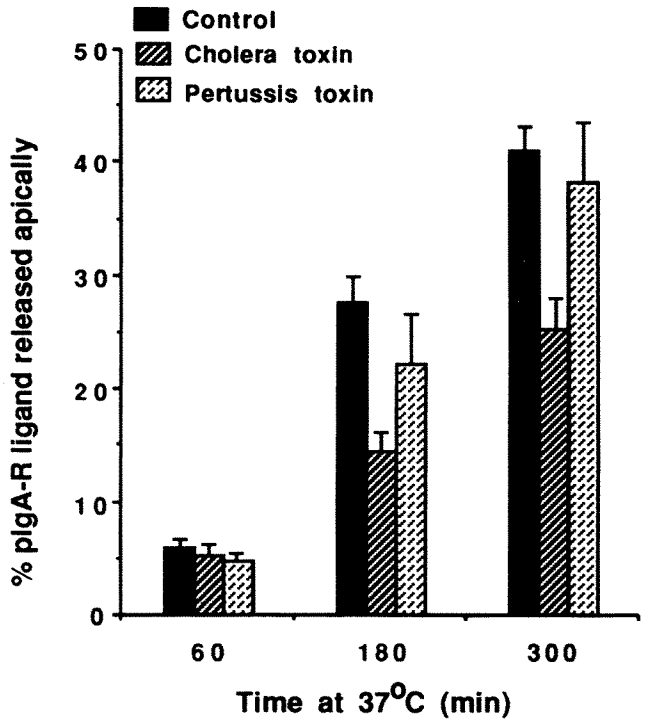


Figure 11. Cholera toxin inhibits late steps of the transcytotic pathway. MDCK cells internalized radiolabeled pIgA-R ligand from the basolateral surface for 4 h at 17°C. (*Control bars*) The cells were then supplemented with nocodazole and incubated at 37°C from different periods of time. (*Cholera toxin bars*) The cells were then supplemented with nocodazole and cholera toxin and incubated at 37°C for 1 h. The cells were then shifted to media containing nocodazole for the next 4 h. (*Pertussis toxin bars*) The cells were then supplemented with nocodazole and pertussis toxin and incubated at 37°C for different periods of time. Percent values represent the amount of radiolabeled pIgA-R ligand released into the apical media, considering the total amount of pIgA-R ligand internalized into the transcytotic pathway as 100%. The bars represent the mean ± SD from 2–3 experiments.

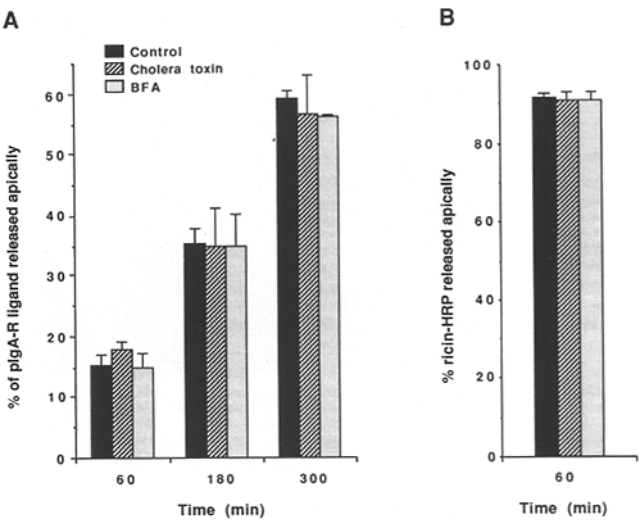


Figure 12. Apical recycling of pIgA-R ligand and ricin are not affected by cholera toxin and BFA. (*A*) MDCK cells internalized apically added radiolabeled pIgA-R ligand for 2 h at 17°C. The cells were then supplemented with nocodazole and incubated at 37°C for various periods of time, in the absence (*control bars*) or in the presence of cholera toxin (*cholera toxin bars*), or BFA (*BFA bars*). Percent values represent the amount of radiolabeled pIgA-R ligand released into the apical media, considering the total amount of internalized pIgA-R ligand as 100%. The bars represent the mean ± SD from 2–3 experiments. (*B*) MDCK cells internalized apically added ricin-HRP for 2 h at 17°C. The cells were then supplemented with nocodazole and incubated at 37°C in the absence (*control bar*) or in the presence of cholera toxin (*cholera toxin bar*) or BFA (*BFA bar*). Percent values represent the amount of ricin-HRP released into the apical media, considering the total amount of internalized ricin-HRP as 100%. The bars represent the mean ± SD from 2–3 experiments.

at 17°C occurs normally, but that the ligand is not released into the apical media. Instead, the ligand accumulates in the sub-apical region of cells, from where it can be chased into the apical media by incubating the cells at 37°C. Addition of nocodazole during the chase dissociates the early transcytotic steps, when cargo is delivered from the basolateral PM to the apical compartment, from the late steps during which transcytosed cargo is delivered from the apical compartment to the apical PM.

pIgA-R ligand internalized from the basolateral surface of MDCK cells grown at 17°C is present in three distinct transport pathways. The *first* pathway contains pIgA-R ligand still present within the basolateral endosome before sorting into TCVs. Within this pathway, the ligand colocalizes with internalized transferrin, a known marker for the basolateral recycling pathway. The ligand will be released into the basolateral media when the cells are shifted to 37°C in the presence of nocodazole since recycling is not microtubule-dependent (Hunziker et al., 1990; Breitfeld et al., 1990). As shown by Breitfeld et al. (1990), inhibition of transcytosis by nocodazole results in increased recycling of pIgA-R ligand to the basolateral PM, presumably as a consequence of saturating the transcytotic pathway sorting mechanism in the basolateral endosome.

The *second* pathway contains ligand already sorted out from the basolateral endosome into TCVs but not yet fully translocated into the apical region. This group will remain inside the cells when cells are shifted to 37°C in the presence of nocodazole since microtubule depolymerization will prevent TCV translocation into the apical region and their return to the basolateral region.

The *third* pathway contains ligand already sorted and translocated into the apical region during the 17°C internalization period. Ligand present within this pathway will be the only one to be released into the apical media when cells are shifted to 37°C in the presence of nocodazole. 42% of the pIgA-R ligand internalized during the 17°C incubation is present within this pathway.

In this study we have used the low temperature block to "trap" transcytosed cargo in the apical region and have characterized this apical compartment by analyzing its accessibility to apically internalized markers such as ricin (a marker for pathways accessible to endocytosed apical PM proteins), pIgA-R ligand (a marker for receptor-mediated apical recycling pathway), and BSA (a fluid-phase marker).

Using double-label fluorescence confocal microscopy, we found ricin to colocalize with the apical transcytotic compartment containing basolaterally internalized pIgA-R ligand. As shown previously (van Deurs et al., 1990; Prydz et al., 1993) using electron microscopy, apically internalized ricin enters an apical endosomal system morphologically characterized by tubular and vesicular sub-domains, and we

assume that the pIgA-R ligand is present within the same ricin-positive compartment. Preliminary results using electron microscopy from our (data not shown) and Mostov's laboratory [Apodaca, G., L. A. Katz, and K. E. Mostov. Receptor mediated transcytosis of IgA in MDCK cells is via apical endosomes. 1993. *Mol. Biol. Cell.* 4:97a.] show colocalization of transcytosing ligand and apically internalized ricin.)

Apically internalized pIgA-R ligand has been shown to recycle back to the apical PM and was used in this study as a marker for the apical receptor-recycling pathway. We found that pIgA-R ligand internalized from the basolateral surface and pIgA-R ligand internalized from the apical surface colocalize in an apical endosomal compartment involved in the apical recycling of membrane-bound proteins (e.g., ricin). Thus, pIgA-R ligands internalized from basolateral and apical surfaces of polarized MDCK cells and present within functionally different pathways (transcytosis vs recycling) meet in the same apically localized endosomal compartment. This meeting occurs before the basolaterally internalized pIgA-R ligand reaches the apical PM and can be reendocytosed, since when anti-ligand antibodies are added apically to act as a marker for apical reendocytosis of basolaterally internalized ligand, no significant internalization of the anti-ligand antibodies is seen at 17°C. Furthermore, when internalization at 17°C proceeds for only 3 h, colocalization of both apically and basolaterally internalized pIgA-R ligand is observed before the transcytosing ligand reaches the top/apical region of the cells.

Since the meeting between transcytotic pIgA-R ligand and apically internalized markers occurs at low (17°C) temperature, it is unlikely that it involves mixing of markers via late endosomal compartments. As shown previously, transport to late endosomes is inhibited under reduced temperature (Dunn et al., 1980; Bomsel et al., 1989), as well as following nocodazole treatment (Gruenberg et al., 1989). Furthermore, loading of late endosomes/lysosomes with fluid phase marker for 1 h at 37°C did not result in colocalization with transcytotic cargo internalized at 17°C.

pIgA-R is not targeted into the lysosomal pathway despite the fact that dimeric Fab (capable of cross-linking) is used as a ligand. Similarly, TGN38 tagged with anti-TGN38 antibodies at the cell surface is correctly targeted to the TGN (Ladinsky and Howell, 1992). It has been suggested (Stanley and Howell, 1993) that molecules like TGN38, which might bind other proteins in the course of their normal intracellular cycling, are not sensitive to perturbation in their transport pathways when tagged with dimeric antibodies. It is therefore likely that receptors like the pIgA-R, which normally bind polyvalent ligands, can move along normal physiological pathway even if tagged with antibodies.

The apical transcytotic compartment, which overlaps with ricin and apically internalized pIgA-R recycling compartment, actively excluded BSA under the 17°C internalization conditions. Rapid separation of cointernalized membrane-bound and fluid phase markers was also observed at 37°C. The mechanism for separating membrane-bound receptors from ligands dispersed in the endosome lumen is probably provided by the geometry of the apical-sorting endosome. As shown by Geuze et al. (1984), the *basolateral* sorting endosome accumulates membrane-bound receptors in its tubular domains, while soluble ligands are mainly found in the vacuolar-like portions. Thus, the recycling receptors rapidly exit the endosome and reach a low steady-state value, while soluble proteins continue to accumulate for a longer period of time and to a greater degree (Mueller and Hubbard, 1986; Dunn et al., 1989; Mayor et al., 1993). It appears that the *apical* endocytic compartment involved in the late steps of the transcytotic pathway and in apical recycling is also composed of tubular and vacuolar compartments, and that the sorting of fluid-phase markers from membrane-bound markers occurs in the tubular regions characterized by a high surface area-to-volume ratio (Tooze and Hollinshead, 1991). The existence of a tubular apical endocytic compartment involved in recycling apically internalized pIgA-R agrees with findings by Hoppe et al. (1985), showing that pIgA-R containing tubules are present in both the basolateral (sinusoidal) and the apical (bile canalicular) regions of hepatocytes although whether these tubules were recycling endosomes was not established in that study. More recently, Quintart et al. (1989) and Barr and Hubbard (1993), showed that pericanalicular vesicles containing pIgA-R are enriched in biliary constituents before their fusion with the bile canaliculi, suggesting that PM vesicles containing pIgA-R fuse with apical endosomes at the canalicular membrane.

It has been proposed that in addition to the presence of distinct sub-domains within a single endosomal compartment, spatially distinct endosomal compartments might exist. Using the basolateral transferrin receptor recycling pathway as a model system, it has been suggested that after sorting within the early endosome, the transferrin receptors exit that compartment and enter a putative recycling endosome which connects the early endosome with the PM (Stoorvogel et al., 1991; Dunn et al., 1989; van der Sluijs et al., 1992; Mueller Hubbard, 1986; Mayor et al., 1993). Whether a similar recycling compartment, separate from the early endosome, exists in the apical region of MDCK cells and what it's relationship to the transcytotic pathway is, remains to be investigated.

We have used specific compounds to analyze transcytotic traffic from the 17°C apical transcytotic compartment to the apical PM.

BFA blocks various membrane transport pathways in a cell-type specific manner, presumably by affecting the balance between vesicle- and tubule-mediated traffic (Lippincot-Schwartz et al., 1990, 1991; Wood et al., 1991; Ladinsky et al., 1992; Apodaca, G., L. A. Katz, and K. E. Mostov. 1993. Receptor mediated transcytosis of IgA in MDCK cells is via apical endosomes. *Mol. Biol. Cell.* 4:97a). Within the transcytotic pathway, Hunziker et al. (1991) have shown that BFA inhibits transcytosis at the level of the basolateral endosome, possibly by blocking the sorting of dIgA-pIgA-R complexes into transcytotic vesicles. We found that BFA also inhibits late steps of the transcytotic pathway, after pIgA-R ligand has been delivered into the apical transcytotic compartment. Significantly, BFA does not affect recycling of membrane-bound markers (ricin and apically internalized pIgA-R ligand) through that same compartment, indicating that membrane flow to and from the apical endosome is normal but that transcytotic cargo is inhibited from entering the recycling pathway. The data suggest that BFA is acting within the apical endosome by inhibiting a normal sorting process necessary for transcytotic cargo inclusion into a recycling membrane, and that BFA action within the apical endosome might be analogous to its role within the basolateral endosome.

Treatment of cells with BFA results in the rapid release of several peripherally associated membrane proteins, for example, β-COP, ARF, and p200 (Donaldson et al., 1991; Narula et al., 1992). β-COP is a component of the coatomer complex found on non-clathrin-coated vesicles involved in ER to Golgi and intraGolgi transport (Waters et al., 1991). ARF has been shown to be critical for the association of coatomers with membranes (Serafini et al., 1991; Donaldson et al., 1992; Palmer et al., 1993) while the role of p200 is currently undefined. BFA also inhibits the membrane binding of at least one other type of coat protein: γ-adaptin which is found in the HA1/AP1 adaptor complex present on *trans*-Golgi network derived clathrin-coated vesicles (Robinson and Kreis, 1992; Wong and Brodsky, 1992). The exact function of coat proteins is not known but it is clear from cell biological studies (Pepperkok et al., 1993) and genetic analyses (sec 21) (Hosobuchi et al., 1992) that they are required for normal membrane traffic. Although rat liver TCVs analyzed in situ do not possess morphologically distinct coats (Renston et al., 1980 and data not shown), biochemical studies on immunoisolated rat liver TCVs indicate that they contain a homoligomeric complex, composed of a 108-kD transcytosis associated protein (TAP), as a major protein component associated with their cytoplasmic surface (Sztul et al., 1991, 1993; Gow et al., 1993). The negative effect of BFA on transcytosis suggests that peripherally associated proteins, similar or distinct from the TAP complex, might be responsible for the inhibition.

It has been shown that activation of trimeric G proteins antagonizes BFA action and promotes coatomer binding to Golgi membranes (for review see Bomsel and Mostov, 1992 and Klausner et al., 1992). More recent studies indicate that BFA's target is probably part of a molecular mechanism regulating ARF function, normally controlled by trimeric G proteins (Randazzo et al., 1993; Helms and Rothman, 1992). Consequently, we have used the MDCK system described above to examine the involvement of trimeric G proteins in the late steps of the transcytotic pathway. The obtained results show that cholera toxin, a known activator of Gsα, has an inhibitory effect on the late steps of the transcytotic pathway by partially blocking the delivery of pIgA-R from the apical endosome into the apical media.

A direct confirmation of a cause-effect relationship between ADP-ribosylation of Gsα by cholera toxin and a reduced transcytotic delivery of pIgA-R ligand into the apical media is difficult to obtain due to the absence of a simple method to study the action of toxin ADP-ribosyltransferases in intact cells (Staddon et al., 1991). Although the predominant effect of cholera toxin treatment of cells is to ADP-ribosylate Gsα proteins and constitutively activate them, cholera toxin can also ADP-ribosylate other intracellular protein(s) (e.g., pertussis toxin sensitive GTPases or tubulin, a 60-kD GTP-binding protein) (Gill and Woolkalis, 1991). Since pertussis toxin has no apparent effect on our in vivo system, activation of Gi or Go proteins is not likely to be involved in the regulation of transcytotic traffic. Similarly, microtubule stabilization can be ruled out as an explanation for the effects induced by cholera toxin since the microtubule network was disrupted before the addition of this compound to the cells. Furthermore, data showing the presence of Gsα proteins in TCVs immunoisolated from rat liver and their ability to be ADP-ribosylated by cholera toxin (Barroso, M., and E. S. Sztul, manuscript in preparation), strongly suggest that Gs proteins are, in fact, responsible for the cholera toxin mediated inhibition of transcytosis.

In addition, back-ribosylation experiments performed in MDCK cells pretreated with cholera toxin showed that 25–30% of the intracellular Gsα population is accessible to ADP-ribosylation by cholera toxin under our treatment conditions (data not shown). These results agree with previously published data which indicate that only a fraction of Gsα proteins is ADP-ribosylated by cholera toxin in fibroblasts and PC12 cells (Watkins et al., 1981; Leyte et al., 1992). One of the effects of cholera toxin-induced activation of Gs is to constitutively activate adenylate cyclase, thereby increasing intracellular levels of cAMP. Experiments with forskolin, a compound known to bypass the trimeric GTPase effect and directly stimulate adenylate cyclase (Seamon and Daly, 1986), suggest that the effect of Gs activation in the in vivo system is not mediated via the cAMP signaling cascade (data not shown).

Cholera toxin and BFA appear to affect specifically the transcytotic traffic of pIgA-R ligand through the apical compartment since other traffic, namely apical recycling, is not inhibited by these reagents. The results are in agreement with the data showing that basolateral recycling of transferrin is not affected by either cholera toxin or BFA treatments (Bomsel and Mostov, 1992). The finding that transcytotic traffic distal to the apical transcytotic compartment is inhibited by cholera toxin and BFA while recycling from that compartment is not, supports our conclusion that the meeting between the transcytotic and apical recycling pathways occurs before fusion with apical PM and thus, does not depend on the apical recycling of the basolaterally internalized pIgA-R ligand. If basolaterally internalized pIgA-R ligand was entering the apical endosome by movement from the apical PM, its recycling to the apical PM (like the recycling of apically internalized pIgA-R ligand) would not be inhibited by BFA and cholera toxin. It appears therefore, that late steps of the transcytotic pathway and apical membrane recycling progress via *the same* endosomal compartment, but that each pathway is regulated by *different* sorting mechanisms which are capable of distinguishing between transcytotic and recycling cargo. These findings also imply that the pIgA-R ligand complexes internalized from the apical PM and delivered to the apical endosome differ in their sorting signals from complexes reaching the apical endosome by transcytosis. Since the ligand is identical in both cases, it is likely that the difference is due to alterations within the pIgA-R. The exact nature of these signals remains to be determined.

Recent reports have demonstrated a stimulatory effect of Gs proteins on distinct steps of vesicular transport. Leyte et al. (1992) showed that cholera toxin mediated activation of AtT20 Gs proteins stimulates the formation of secretory vesicles from the TGN, while Pimplikar and Simons (1993) found that activation of MDCK cell Gs proteins stimulates transport of influenza virus HA protein from the TGN to the apical surface. However, in a cell-free endosome–endosome fusion assay mastoparan (which increases the nucleotide exchange by G proteins and results in their activation) inhibits endosomal fusion (Colombo et al., 1992). Similarly, in a cell-free transcytotic assay reconstituting the basolateral endosomal sorting step, Gs proteins in their assumed inactive state (Gs-GDP) are necessary for the formation of TCVs

(Bomsel and Mostov, 1992, 1993). This finding is consistent with ours, since both results indicate that Gs proteins in the activated state (either bound with GTPγS or ADP-ribosylated by cholera toxin) should inhibit sorting of transcytosing pIgA-R ligand within the endosomal compartments (basolateral or apical) of the transcytotic pathway. Whether the effect we observe is due to missorting or to inhibition of vesicle formation from the apical endosome can not be currently ascertained, but the fact that apical recycling is not affected by cholera toxin treatment suggests that sorting rather than membrane traffic is inhibited.

Our finding that cholera toxin treatment has a negative effect on the late steps of the transcytotic pathway represents the first instance where an inhibitory effect of Gs activation on vesicular traffic is reported. Although previous studies have shown that activation of Gs proteins results in the stimulation of membrane traffic, the specific transport step that was analyzed was budding from the TGN. Based on our and others data, it appears that function of Gs in controlling vesicular traffic varies depending on the subcellular compartment and that conclusions based on results from a single compartment should not be generalized to all membrane traffic pathways.

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